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Isolation of Avian Paramyxovirus 1 from a Patient with a Lethal Case of Pneumonia▽

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An unknown virus was isolated from a lung biopsy sample and multiple other samples from a patient who developed a lethal case of pneumonia following a peripheral blood stem cell transplant. A random PCR-based molecular screening method was used to identify the infectious agent as avian paramyxovirus 1 (APMV-1; a group encompassing Newcastle disease virus), which is a highly contagious poultry pathogen that has only rarely been found in human infections. Immunohistochemical analysis confirmed the presence of APMV-1 antigen in sloughed alveolar cells in lung tissue from autopsy. Sequence from the human isolate showed that it was most closely related to virulent pigeon strains of APMV-1. This is the most completely documented case of a systemic human infection caused by APMV-1 and is the first report of an association between this virus and a fatal disease in a human.

The causes of many acute respiratory infections in humans remain undiagnosed, although a significant fraction of these infections are thought to have a viral etiology (4, 29). The past decade has seen the identification of a number of previously unrecognized human respiratory pathogens. Modern molecular virological methods have led to the discovery of the paramyxovirus human metapneumovirus (29), the human coronaviruses NL-63 (30) and HKU1 (33), and the parvovirus human bocavirus (4). Some of these discoveries were stimulated by the dramatic emergence of severe acute respiratory syndrome (18, 23), which was found to be caused by a zoonotically transmitted coronavirus that had evolved the capability for human-to-human transmission. H5N1 and other subtypes of avian influenza virus have also sporadically crossed species barriers (14), although they have not yet demonstrated the capacity to spread by a human-to-human route. This report describes the isolation and identification of avian paramyxovirus 1 (APMV-1) from a lethal case of human pneumonia. APMV-1, which historically was called Newcastle disease virus (NDV), has previously been associated almost exclusively with disease in avian species.

A 42-year-old man who had a history of non-Hodgkin's lymphoma developed a fever and progressive pulmonary infiltrates on broad-spectrum antibiotics and double-antifungal therapy 18 days after receiving a peripheral blood stem cell transplant from a fully matched unrelated donor, following preparation with a nonmyeloablative regimen. The patient initially underwent a bronchoscopy and then an open lung biopsy to establish the etiology of his pneumonia. He died of respiratory failure 24 days following the onset of the infection. Virus isolation was carried out on a number of specimens taken from the patient at various times from days 1 through 21 after the onset of infection. These specimens, which included bronchial wash, bronchial lavage, lung biopsy, stool, and urine samples, were all culture positive, producing cytopathic effects in MRC-5 adult human lung cells, WI-38 embryonic human lung cells, A549 human lung adenocarcinoma cells, and primary rhesus monkey kidney (PRMK) cells. However, in both immediate and subsequent testing for respiratory pathogens, the infectious agent proved refractory to identification. By various combinations of direct or indirect fluorescent-antibody assays or enzyme immunoassays, samples were found negative for the following viruses: adenovirus, influenza viruses A and B, human parainfluenza viruses types 1 through 4, respiratory syncytial virus, measles virus, and mumps virus. Negative results were also obtained in PCR tests for human metapneumovirus and for mycoplasm. By contrast, a positive result that indicated hemadsorption with guinea pig red blood cells was obtained, suggesting that the putative virus contained a hemagglutinin.

Examination of one tissue culture sample by negative-staining electron microscopy revealed very few virus-like particles. One of the best resolved of these (Fig. 1A) was ovoid (100 by 120 nm) and was decorated with a series of projections extending 8 to 10 nm from the virion surface, morphological features consistent with either an orthomyxovirus or a paramyxovirus. More frequently observed were filamentous structures having the very characteristic profiles of helical viral nucleocapsids (Fig. 1B and C). The clear "herringbone" patterns of these ribonucleoproteins, their

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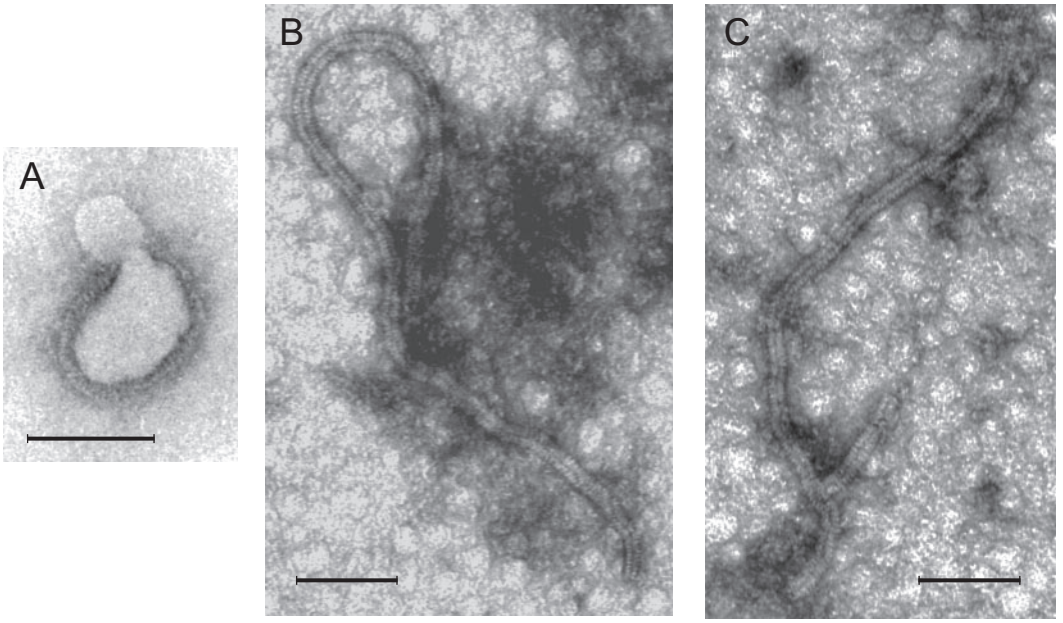

FIG. 1. Electron micrographs of infected PRMK cell culture supernatant: a virion (A) and viral nucleocapsids (B and C). Samples were adsorbed onto Formvar carbon 400-mesh-coated copper grids (Electron Microscopy Sciences) and were negatively stained with either 2% sodium phosphotungstate (pH 7.0) or 0.5% uranyl acetate (pH 7.0). Samples were viewed under a Zeiss (LEO) 910 transmission electron microscope operating at 80 KeV, and images were recorded at a magnification of ×25,000. Bars denote 100 nm.

diameters (17 to 20 nm), and the diameters of their central holes (3.5 to 4 nm) are defining features of paramyxovirus nucleocapsids. Additionally, the filament lengths (850 to 1,100 nm) were consistent with the sizes of paramyxovirus nucleocapsids but the filaments were far longer than those of orthomyxoviruses. This finding was paradoxical, since all paramyxoviruses commonly associated with human infections had been ruled out by clinical diagnostic assays.

Given the apparently contradictory nature of some of the evidence, a molecular biological method was designed to allow determination of the identity of the viral agent without making prior taxonomic assumptions. Random screening methods have been used in a number of recent instances to detect novel viruses (3, 4, 29, 30). The procedure that we developed (Fig. 2A) combined elements of a previously reported random cloning method (15) with a nuclease pretreatment step (3) to remove contaminating cellular material. Micrococcal nuclease was used because it hydrolyzes both RNA and DNA and because it is inactivated by chelation of calcium ions (2). The cloning scheme was intentionally kept relatively simple, avoiding elaborations such as adapter ligation or anchored primers, in order to increase efficiency by reducing accumulated sample loss that might have occurred over multiple steps. The procedures were also designed to apply equally well to RNA or DNA as starting material.

For 18 random clones, with inserts of 150 to 1,400 bp, sequences were determined and identified by BLAST searches against the GenBank database (5). Twelve of the clone inserts exhibited 88% to 99% identity with primate chromosomal sequences, indicating that they originated from the PRMK cells in which the virus had been cultured. All six of the remaining clones had inserts that were 93% to 98% identical to strains of the paramyxovirus APMV-1 (Fig. 2B). This result, while consistent with the previous electron microscopic observations, was wholly unexpected, since APMV-1 typically infects only avian hosts. In this regard, it should be noted that APMV-1 has never been present in any of the laboratories that carried out the virus isolation or cloning in this study. The six insert sequences mapped to multiple sites across the APMV-1 genome, covering 21% of the total genome, and showed the highest sequence homology to strains of this virus that had previously been isolated from pigeons in Europe and North America (6, 22, 27, 28, 32).

To further corroborate APMV-1 as the infectious agent, PCR primers were designed for a diagnostic assay for the F gene that was applied to RNA purified from multiple virus isolation samples from the infected patient. This test revealed that, for every viral culture derived from specimens taken on days 1 through 21, the target sequence was amplified by reverse transcription-PCR (Fig. 3). The specificity of the assay for the APMV-1 F gene was confirmed through sequencing of all resulting PCR products; in addition, no PCR products of any size were obtained with total RNA from uninfected cells. The presence of APMV-1 in multiple respiratory samples, as well as in urine and stool samples, suggested that the patient's infection had been systemic. Additional confirmation of the identity of the virus was provided by a Western blot of lysates from infected cell cultures that were probed with chicken polyclonal anti-NDV antiserum (SPAFAS Avian Products, Charles River Laboratories), which revealed APMV-1-specific proteins that were not present in uninfected cells (data not shown).

Patient lung samples taken at autopsy were examined for pathology and for the presence of virus. Histopathologic

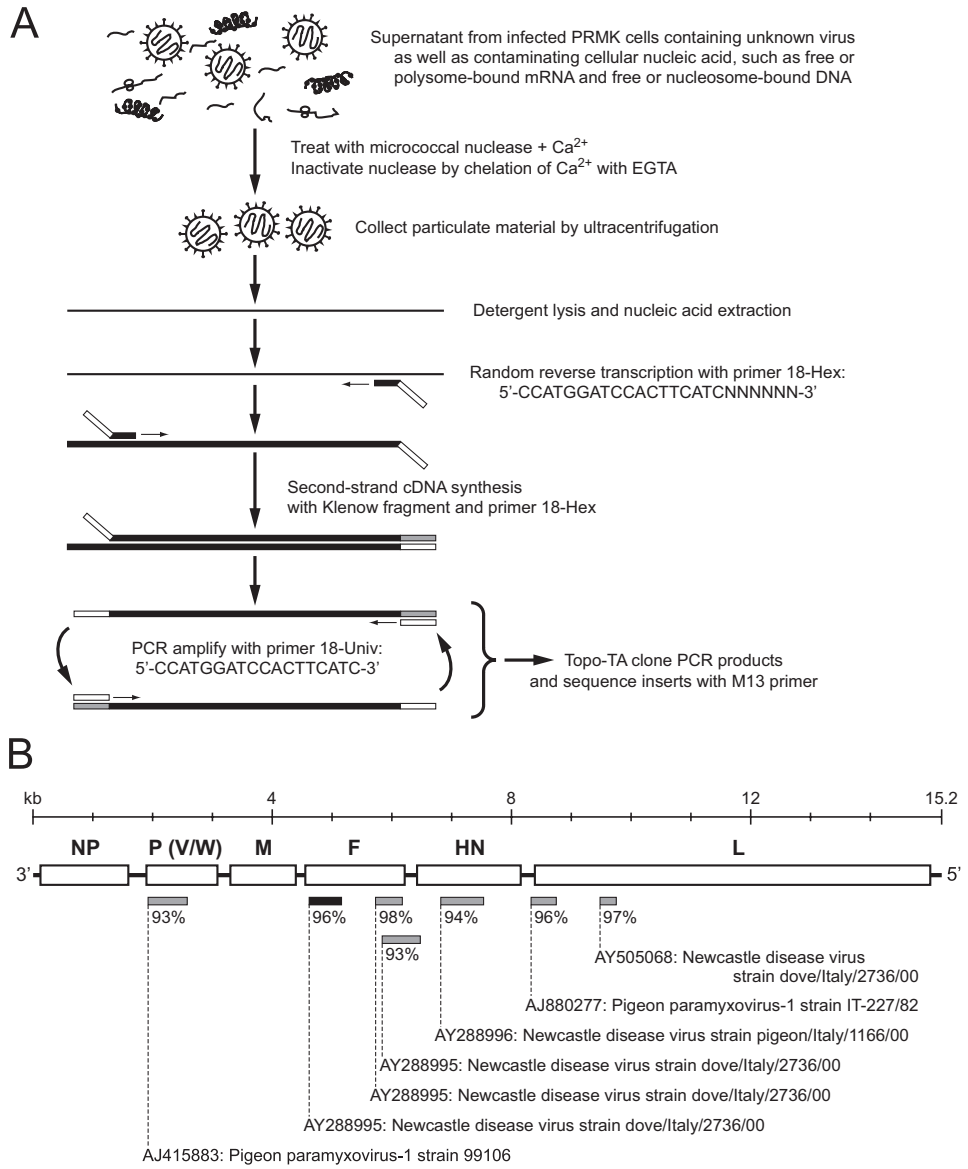

FIG. 2. Identification of the infectious agent as APMV-1. (A) Schematic of a strategy for random cloning of nucleic acid from an unknown virus. Virus cultured from the day 11 lung biopsy specimen was amplified in PRMK cells. Clarified tissue culture supernatant was incubated with 20 U/ml micrococcal nuclease (Worthington Biochemical) to digest soluble material from lysed cells, after which the reaction was quenched by the addition of EGTA and EDTA. Viral particles were collected by ultracentrifugation, and nuclease-protected genomes were purified by phenol and chloroform extraction, followed by ethanol precipitation. First-strand cDNA synthesis was carried out with avian myeloblastosis virus reverse transcriptase (Life Sciences), using a random hexamer primer, 18-Hex, which also contained a 5′ extension of defined sequence. Second-strand cDNA synthesis was carried out with a DNA polymerase Klenow fragment (New England Biolabs) and the same primer. Resulting cDNA species were then amplified by PCR with a universal primer, 18-Univ, identical to the defined portion of 18-Hex. PCR products were cloned in bulk with a Topo-TA kit (Invitrogen). A detailed protocol is available upon request. (B) Loci of random clone sequences. The negative-stranded APMV-1 genome is represented in the 3′-to-5′ direction, with rectangles indicating (positive-sense) open reading frames for the nucleoprotein (NP), phosphoprotein (P), P gene editing products (V and W), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L). Gray rectangles denote the locations and sizes of sequences obtained from random clone inserts. The black rectangle represents a PCR product sequence obtained from a region of the F gene. Indicated beneath each rectangle are the percent sequence identity, accession number, and viral isolate source of the most closely matching GenBank entry.

examination of a section of lung (Fig. 4A and B) revealed a subacute, severe, diffuse interstitial pneumonia with flooding of alveoli by fibrin, protein-rich fluid, red blood cells, sloughed pneumocytes, and a few neutrophils. Perivascular edema around a large vessel, as well as abundant black granular pigment (inhaled dust, an incidental finding), was also noted. Immunohistochemical staining of patient lung samples was carried out with a monoclonal antibody specific

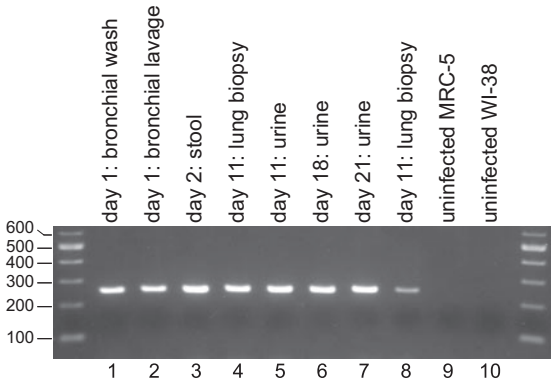

FIG. 3. Reverse transcription-PCR analysis of virus cultured from successive specimens from the infected patient. The time at which the first specimen was taken for virus isolation is defined as day 1. A fragment of the F gene of the human APMV-1 isolate, separate from those segments originally cloned, was amplified with primers based on the most closely matching GenBank database sequence. Sequence obtained therefrom was then used to design a diagnostic primer pair, N-7 (5′-TGACGAGCTCTCTTGACGGC-3′) and N-12 (5′-CCTCCTGATGTGGACACAGA-3′), to amplify a 239-bp target for analysis of clinical samples. RNA purified from tissue culture supernatants of clinical samples was reverse transcribed with a random hexanucleotide primer and amplified in PCRs with primers N-7 and N-12 run for 30 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. PCR product sequences were verified with primer N-8 (5′-ATTGTAGTGACAGGAGATA-3′). Analyzed samples in lanes 1 through 7 had been cultured in MRC-5 cells; the sample in lane 8 had been cultured in WI-38 cells. Samples in lanes 9 and 10 were uninfected cell controls; sizes of DNA standards in flanking lanes are indicated in base pairs.

for APMV-1 P protein (kindly provided by Mark E. Peeples). This staining showed widespread, positively reacting cells, which appeared to be mostly sloughed pneumocytes, throughout the section (Fig. 4C). Similarly labeled cells were observed in sections of tissue from birds infected with APMV-1 (Fig. 4E and F). No labeling was seen in a control sample of the patient lung stained with an irrelevant primary antibody (Fig. 4D), and no reactivity was detected in avian and mammalian tissues infected with a number of other pathogenic viruses, bacteria, or protozoal or fungal organisms (not shown). Likewise, the anti-APMV-1 monoclonal did not stain a control sample of human lung infected with *Pneumocystis carinii* (not shown). These findings strongly argue that infection with APMV-1 accounted for the histopathologic finding of severe interstitial pneumonia.

The identification of APMV-1 as the infectious agent was surprising, since this virus is not considered a cause of serious disease in humans. Newcastle disease emerged in the early 20th century as a rapidly spreading, highly pathogenic disease of poultry (1). Cases of human infection with APMV-1 are rare and have been reviewed periodically (9, 16, 25, 26). It is well established that APMV-1 can cause an acute and rapidly clearing conjunctivitis, the first example of which was reported by Macfarlane Burnet as resulting from a laboratory accident in 1942 (8). Numerous subsequent cases of ocular infection, occasionally accompanied by low fever and chills, were clearly documented as attributable to APMV-1 on the basis of virus isolation and serology (11, 16, 24). Such cases almost always occurred in poultry workers preparing or administering a lyophilized or aerosolized NDV vaccine. By contrast, the association of APMV-1 with human respiratory disease has been more tenuous. Early reports of human APMV-1 infection or its relationship to influenza-like symptoms that were based solely on serology may be ascribed to cross-reactivity with antibodies to mumps or parainfluenza viruses (7, 16, 17, 25). One early communication claimed the possible recovery of NDV from lung biopsy material from a patient with pneumonia (7); however, that report lacked data and contained conflicting results, and its conclusions were subsequently questioned (13). The data presented here make it clear that, under particular circumstances, it is indeed possible for APMV-1 to cause severe human respiratory disease. To our knowledge, this is the most completely documented report of a case of pneumonia, or any systemic human infection, caused by APMV-1. Additionally, it is the first report of an association between this virus and a fatal disease in a human.

The sequenced genomic segments in the current study (Fig. 2B) place the infecting virus among strains of APMV-1 that have been isolated from domesticated and urban pigeons and doves (6, 22, 27, 28, 32). This finding suggests, but does not prove, a pigeon origin for the patient's infection. The patient was an urban dweller, but it is not known whether he had pets or was exposed to avian species in other settings. However, the ubiquity of pigeons in urban areas certainly makes them a feasible source. Moreover, APMV-1 survives well under a variety of environmental conditions (16). The virus is stable in bird feces and can be spread via direct contact or by wind-borne dust (31). As in many pigeon strains of APMV-1, the deduced F protein cleavage site of the human isolate of the virus, 110-GGRRKKRFIG-119, contains five consecutive lysine or arginine residues. Such a basic cleavage site is characteristic of the most virulent strains of APMV-1 because of the ease with which the F preprotein can be cleaved to its active, fusogenic form by furin or other cellular proteases (12, 21, 22).

One other potential mechanism of human infection with APMV-1 must be noted: this virus has been used for a considerable time as an oncolytic agent in experimental cancer therapy for solid tumors. Trial studies administering APMV-1 by intravenous injection or inhalation have been carried out for at least 2 decades with no significant adverse effects reported (10, 20, 26). The patient in the present case was not a participant in any such study. Moreover, sequence information from the current isolate sets it apart from the attenuated strain of APMV-1 that is used in clinical trials in the United States (19).

In conclusion, the APMV-1-associated pneumonia reported here was likely to have been a unique case, probably an opportunistic infection due to the patient's underlying medical condition. However, this case shows that physicians and diagnostic laboratories should consider APMV-1 a possible source of respiratory disease, especially in light of the significant segment of the population that is immunosuppressed owing to human immunodeficiency virus infection, cancer chemotherapy, or organ transplantation.

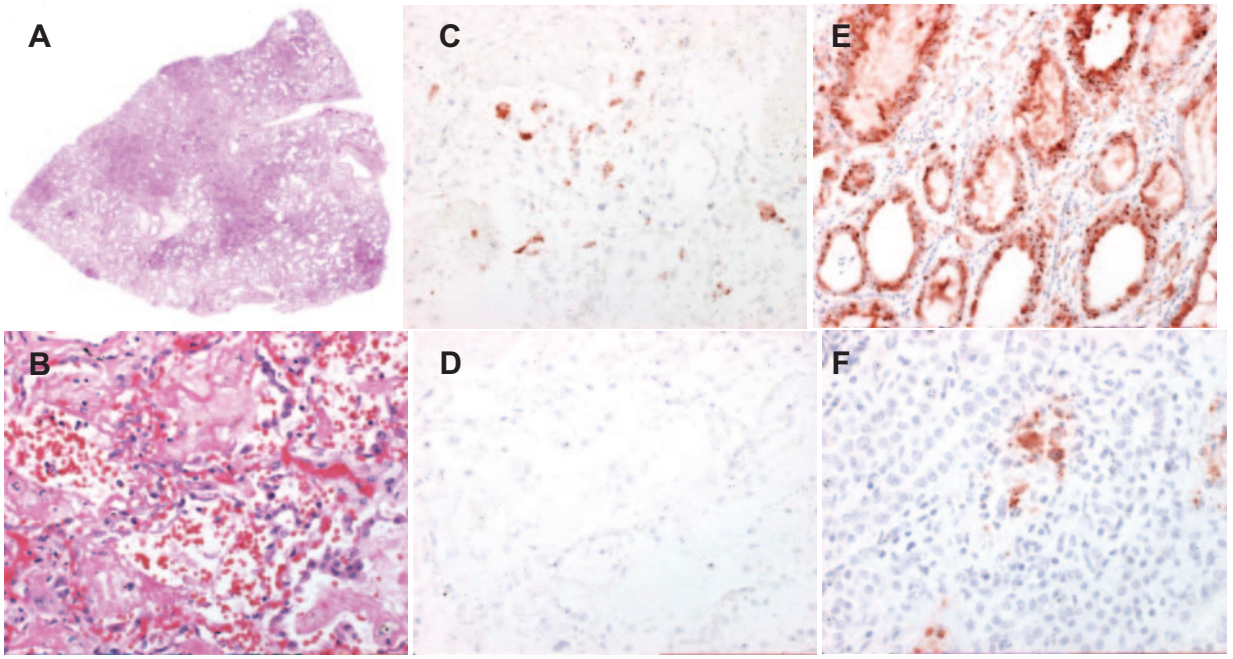

FIG. 4. Pathology and immunohistochemistry. (A) Low-magnification image of a sectioned, paraffin-embedded patient lung sample, showing many alveoli containing protein and blood. Staining was with hematoxylin and eosin. (B) Alveolar parenchyma of a patient lung sample showing severe interstitial pneumonia: the alveolar spaces are filled with red blood cells, laminated eosinophilic protein, and rare inflammatory cells and are bordered by detached, possibly sloughed, low cuboidal type II pneumocytes. Black granular inhaled dust at lower right and upper left is incidental. Hematoxylin and eosin staining was used. Magnification, ×200. (C) Immunohistochemical staining of a patient lung sample, using a monoclonal antibody directed against APMV-1 P protein, showing positively reacting cells that resemble sloughed pneumocytes in diseased alveoli. Hematoxylin counterstaining was used. Magnification, ×200. (D) Negative control for immunohistochemical staining of patient lung sample, using an irrelevant primary antibody (mouse immunoglobulin G) and the same labeled secondary antibody as in panel C. No reactivity was found in any cells. Hematoxylin counterstaining was used. Magnification, ×200. (E) Positive control for immunohistochemical staining. Proventricular glands of an APMV-1-infected chicken stained in the same manner as in panel C, with epithelial cells exhibiting strong, diffuse reactivity with anti-APMV-1 antibody. Hematoxylin counterstaining was used. Magnification, ×200. (F) Positive control for immunohistochemical staining. A kidney of an APMV-1-infected pigeon, stained in the same manner as in panel C, showing sloughed tubular epithelial cells reactive with anti-APMV-1 antibody. Hematoxylin counterstaining was used. Magnification, ×400. For immunohistochemical staining (C to F), deparaffinized slides were rinsed in 100% ethanol, quenched with 3% hydrogen peroxide in absolute methanol, and then rinsed successively in 95% ethanol, 70% ethanol, and deionized water. They were treated with an antigen retrieval solution consisting of 0.001% protease (protease type XIV; Sigma) in 1× target retrieval solution (Dako) for 40 min at 96°C, cooled, and rinsed in deionized water followed by Tris-buffered saline–Tween buffer. Slides were then treated with 3% normal horse serum for 30 min, and primary antibody was applied at a dilution of 1:50 for 60 min at room temperature. Following rinsing in Tris-buffered saline–Tween, slides were incubated for 30 min with a labeled polymer (Dako EnVision+ horseradish peroxidase), rinsed in Tris-buffered saline–Tween, and treated with 3-amino-9-ethylcarbazole (Dako) for 10 to 20 min. The slides were then rinsed in deionized water, counterstained with Mayer's hematoxylin, and coverslipped using Crystal Mount (Biomedia).

Nucleotide sequence accession numbers. The APMV-1 cDNA sequences have been deposited in GenBank under accession numbers EF555090 to EF555096.

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